

NEW METHODS FOR ARRAY PREPARATION USING SUBSTRATE ROTATION

CROSS-REFERENCES TO RELATED APPLICATIONS

5 This application claims the benefit of 60/209,411, filed June 1,
2000, the disclosure of which is incorporated by reference.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

10 Not applicable

BACKGROUND OF THE INVENTION

15 The present invention relates to improved methods for preparing support-
bound nucleic acid arrays. More particularly, the invention relates to methods of
preparing the arrays wherein a planar array substrate is held in a substantially vertical
position and rotated during the course of nucleic acid synthesis.

20 Substrate-bound nucleic acid arrays, such as the Affymetrix DNA Chip,
enable one to test hybridization of a target nucleic acid molecule to many thousands of
differently sequenced nucleic acid probes at feature densities greater than about five
hundred per 1 cm². Because hybridization between two nucleic acids is a function of
their sequences, analysis of the pattern of hybridization provides information about the
sequence of the target molecule. The technology is useful for *de novo* sequencing and re-
sequencing of nucleic acid molecules and also has important diagnostic uses in
25 discriminating genetic variants that may differ in sequence by one or a few nucleotides.
For example, substrate-bound nucleic acid arrays are useful for identifying genetic
variants of infectious diseases, such as HIV, or genetic diseases, such as cystic fibrosis.

30 In one version of the substrate-bound nucleic acid array, the target nucleic
acid is labeled with a detectable marker, such as a fluorescent molecule. Hybridization
between a target and a probe is determined by detecting the fluorescent signal at the
various locations on the substrate. The amount of signal is a function of the thermal
stability of the hybrids. The thermal stability is, in turn, a function of the sequences of the
target-probe pair: AT-rich regions of DNA melt at lower temperatures than GC-rich

regions of DNA. This differential in thermal stabilities is the primary determinant of the breadth of DNA melting transitions, even for nucleic acids.

Depending upon the length of the nucleic acid probes, the number of different probes on a substrate, the length of the target nucleic acid, and the degree of hybridization between sequences containing mismatches, among other things, a hybridization assay carried out on a substrate-bound nucleic acid array can generate thousands of data points of different signal strengths that reflect the sequences of the probes to which the target nucleic acid hybridized. This information can require a computer for efficient analysis. The fact of differential fluorescent signal due to differences in thermal stability of hybrids complicates the analysis of hybridization results, especially from combinatorial nucleic acid arrays for *de novo* sequencing and custom nucleic acid arrays for specific re-sequencing applications. Modifications in custom array designs have contributed to simplifying this problem.

Further complications can arise and lead to variability in diagnostic or sequencing results. For example, degradation of nucleic acid probes, either during the synthesis steps or on standing can lead to variability in assay results. Accordingly, there exists a need for additional methods of nucleic acid array preparation, and the arrays themselves, to provide more robust tools for the skilled researcher. The present invention provides such methods and arrays.

SUMMARY OF THE INVENTION

The present invention provides an improved method for forming nucleic acid arrays, or more generally, any oligomeric arrays. In a number of array fabrication technologies, the substrate on which synthesis takes place is held in a substantially vertical position. Once regions of the substrate have been activated, a suitable monomer (typically in solution) is contacted with the substrate for attachment to the nascent oligomer. Underlying the present invention was the discovery that lower portions of arrays, in some instances, yielded suboptimal products. Accordingly, in a fabrication process on a substrate or wafer divided into 49 chips (a 7 x 7 chip), there was a large intra wafer variation between the top and bottom probe arrays. Surprisingly, by rotating the substrate (or wafer) during array synthesis, essentially all chips produced passed the stringent quality control criteria.

In view of the above, the present invention provides a method of preparing a nucleic acid array on a support, wherein each nucleic acid occupies a separate known region of the support, said synthesizing comprising:

(a) activating a region of the support;

(b) attaching a nucleotide to a first region, said nucleotide having a masked reactive site linked to a protecting group;

(c) repeating steps (a) and (b) on other regions of said support whereby each of said other regions has bound thereto another nucleotide comprising a masked reactive site link to a protecting group, wherein said another nucleotide may be the same or different from that used in step (b);

(d) removing the protecting group from one of the nucleotides bound to one of the regions of the support to provide a region bearing a nucleotide having an unmasked reactive site;

(e) binding an additional nucleotide to the nucleotide with an unmasked reactive site;

(f) repeating steps (d) and (e) on regions of the support until a desired plurality of nucleic acids is synthesized, each nucleic acid occupying separate known regions of the support;

wherein the surface of said substrate is maintained in a position which is vertical or within about 30 degrees of vertical during the attaching and binding steps, and

wherein the substrate is rotated around an axis perpendicular to said surface by an amount of from about 20 degrees to about 180 degrees, said rotating being done prior to, coincident with or subsequent to at least one of said attaching or binding steps.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the rotation positions for a wafer during array preparation.

Figure 2 is a graph that illustrates the average difference in control versus rotated arrays.

Figure 3 is a graph that illustrates the average differences for a control wafer (296) versus the rotated wafers (297 and 299).

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Definitions

The following definitions are set forth to illustrate and define the meaning and scope of the various terms used to describe the invention herein.

5 "Nucleic acid library" or "array" is an intentionally created collection of nucleic acids which can be prepared either synthetically or biosynthetically and screened for biological activity in a variety of different formats (*e.g.*, libraries of soluble molecules; and libraries of oligos tethered to resin beads, silica chips, or other solid supports). Additionally, the term "array" is meant to include those libraries of nucleic acids which
10 can be prepared by spotting nucleic acids of essentially any length (*e.g.*, from 1 to about 1000 nucleotide monomers in length) onto a substrate. The term "nucleic acid" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides, that comprise purine and pyrimidine bases, or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases. The
15 backbone of the polynucleotide can comprise sugars and phosphate groups, as may typically be found in RNA or DNA, or modified or substituted sugar or phosphate groups. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. The sequence of nucleotides may be interrupted by non-nucleotide components. Thus the terms nucleoside, nucleotide, deoxynucleoside and
20 deoxynucleotide generally include analogs such as those described herein. These analogs are those molecules having some structural features in common with a naturally occurring nucleoside or nucleotide such that when incorporated into a nucleic acid or oligonucleoside sequence, they allow hybridization with a naturally occurring nucleic acid sequence in solution. Typically, these analogs are derived from naturally occurring
25 nucleosides and nucleotides by replacing and/or modifying the base, the ribose or the phosphodiester moiety. The changes can be tailor made to stabilize or destabilize hybrid formation or enhance the specificity of hybridization with a complementary nucleic acid sequence as desired.

"Solid support", "support", and "substrate" are used interchangeably and
30 refer to a material or group of materials having a rigid or semi-rigid surface or surfaces. In many embodiments, at least one surface of the solid support will be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different compounds with, for example, wells, raised regions, pins, etched

trenches, or the like. According to other embodiments, the solid support(s) will take the form of beads, resins, gels, microspheres, or other geometric configurations.

"Predefined region" or "preselected region" refers to a localized area on a solid support which is, was, or is intended to be used for formation of a selected molecule and is otherwise referred to herein in the alternative as a "selected" region, a "known" region, or a "known" location. The predefined or known region may have any convenient shape, *e.g.*, circular, rectangular, elliptical, wedge-shaped, etc. For the sake of brevity herein, "predefined regions" are sometimes referred to simply as "regions." In some embodiments, a predefined or known region and, therefore, the area upon which each distinct compound is synthesized is smaller than about 1 cm² or less than 1 mm². Within these regions, the molecule synthesized therein is preferably synthesized in a substantially pure form. In additional embodiments, a known region can be achieved by physically separating the regions (*i.e.*, beads, resins, gels, etc.) into wells, trays, etc. Accordingly, materials (*e.g.*, nucleic acids) can be synthesized or attached to any particular region by any known methods or means.

General

Nucleic acid arrays having single-stranded nucleic acid probes have become powerful research tools for identifying and sequencing new genes. Other arrays of unimolecular double-stranded DNA have been developed which are useful in a variety of screening assays and diagnostic applications (see, for example, U.S. Patent No. 5,556,752). Still other arrays have been described in which a ligand or probe (a peptide, for example), is held in a conformationally restricted position by two complementary nucleic acids, at least one of which is attached to a support. Common to each of these types of arrays is the presence of a support-bound nucleic acid and the exquisite sensitivity exhibited by the arrays. Unfortunately, the sensitivity of these arrays can be compromised if the nucleic acids are degraded or are not prepared in sufficient quantity on the support.

In order to provide the researcher with arrays of uncompromising quality and reproducible performance, arrays should be prepared using high yield reactions and excluding any component which could negatively impact synthesis yield or the performance of the array.

The present invention derives from the discovery that improved yields can be obtained if nucleic acid array substrates are held in a substantially vertical position and rotated during and/or between synthesis steps of nucleic acid array preparation.

5 **Embodiments of the Invention**

In view of the above discoveries, the present invention provides an improved method of preparing a nucleic acid array on a support. In a general sense, the method comprises synthesizing a plurality of nucleic acids on a support wherein the
10 support is held in a substantially vertical position and rotated about an axis perpendicular to the substrate surface during the course of synthesis.

Synthesis of Nucleic acid Arrays

15 In the present invention, nucleic acid arrays can be prepared using a variety of synthesis techniques directed to high-density arrays of nucleic acids on solid supports. In brief, the methods can include light-directed methods, flow channel or spotting methods, pin-based methods, or combinations thereof. For light-directed methods, see, for example, U.S. Patent No. 5,143,854, 5,424,186 and 5,510,270. For
20 techniques using mechanical methods, see PCT No. 92/10183, U.S. Patent No. 5,384,261 and PCT/US99/00730. For a description of pin-based methods, see U.S. Patent No. 5,288,514. A brief description of these methods is provided below. The methods of the present invention are equally amenable to the preparation of unimolecular double-stranded DNA arrays (see U.S. Patent No. 5,556,752). In addition, the nucleic acid arrays
25 prepared in the present methods will also include those arrays in which individual nucleic acids are interrupted by non-nucleotide portions (see, for example U.S. Patent No. 5,556,752 in which probes such as polypeptides are held in a conformationally restricted manner by complementary nucleic acid fragments).

 Various additional techniques for large scale polymer synthesis are known.
30 Some examples include the U.S. Patents Nos.: 5,143,854, 5,242,979, 5,252,743, 5,324,663, 5,384, 261, 5,405,783, 5,412,087, 5,424,186, 5,445,934, 5,451,683, 5,482,867, 5,489,678, 5,491,074, 5,510,270, 5,527,681, 5,550,215, 5,571,639, 5,593,839, 5,599,695, 5,624,711, 5,631,734, 5,677,195, 5,744,101, 5,744,305,

5,753,788, 5,770,456, 5,831,070, and 5,856,011, all of which are incorporated by reference herein.

Libraries on a Single Substrate

Light-Directed Methods

For those embodiments using a single solid support, the nucleic acids of the present invention can be formed using techniques known to those skilled in the art of polymer synthesis on solid supports. Preferred methods include, for example, "light directed" methods which are one technique in a family of methods known as VLSIPS™ methods. The light directed methods discussed in U.S. Patent No. 5,143,854 involve activating known regions of a substrate or solid support and then contacting the substrate with a preselected monomer solution. The known regions can be activated with a light source, typically shown through a mask (much in the manner of photolithography techniques used in integrated circuit fabrication). Other regions of the substrate remain inactive because they are blocked by the mask from illumination and remain chemically protected. Thus, a light pattern defines which regions of the substrate react with a given monomer. By repeatedly activating different sets of known regions and contacting different monomer solutions with the substrate, a diverse array of nucleic acids is produced on the substrate. Of course, other steps such as washing unreacted monomer solution from the substrate can be used as necessary.

The VLSIPS™ methods are preferred for the methods described herein. Additionally, the surface of a solid support, optionally modified with spacers having photolabile protecting groups such as NVOC and MeNPOC, is illuminated through a photolithographic mask, yielding reactive groups (typically hydroxyl groups) in the illuminated regions. A 3'-O-phosphoramidite activated deoxynucleoside (protected at the 5'-hydroxyl with a photolabile protecting group) is then presented to the surface and chemical coupling occurs at sites that were exposed to light. Following capping, and oxidation, the substrate is rinsed and the surface illuminated through a second mask, to expose additional hydroxyl groups for coupling. A second 5'-protected, 3'-O-phosphoramidite activated deoxynucleoside is presented to the surface. The selective photodeprotection and coupling cycles are repeated until the desired set of nucleic acids is produced. Alternatively, an oligomer of from, for example, 4 to 30 nucleotides can be added to each of the known regions rather than synthesize each member in a monomer by

monomer approach. Methods for light-directed synthesis of DNA arrays on glass substrates are also described in McGall *et al.*, *J. Am. Chem. Soc.*, **119**:5081-5090 (1997).

For the above light-directed methods wherein photolabile protecting groups and photolithography are used to create spatially addressable parallel chemical synthesis of a nucleic acid array (see also U.S. Patent No. 5,527,681), computer tools may be used to assist in forming the arrays. For example, a computer system may be used to select nucleic acid or other polymer probes on the substrate, and design the layout of the array as described in, for example, U.S. Patent No. 5,571,639.

Flow Channel or Spotting Methods

Additional methods applicable to library synthesis on a single substrate are described in U.S. Pat. No. 5,384,261 and in PCT/US99/00730. In the methods disclosed in this patent and PCT publication, reagents are delivered to the substrate by either (1) flowing within a channel defined on known regions or (2) "spotting" on known regions. However, other approaches, as well as combinations of spotting and flowing, may be employed. In each instance, certain activated regions of the substrate are mechanically separated from other regions when the monomer solutions are delivered to the various reaction sites.

A typical "flow channel" method applied to the compounds and libraries of the present invention can generally be described as follows. Diverse nucleic acid sequences are synthesized at selected regions of a substrate or solid support by forming flow channels on a surface of the substrate through which appropriate reagents flow or in which appropriate reagents are placed. For example, assume a monomer "A" is to be bound to the substrate in a first group of selected regions. If necessary, all or part of the surface of the substrate in all or a part of the selected regions is activated for binding by, for example, flowing appropriate reagents through all or some of the channels, or by washing the entire substrate with appropriate reagents. After placement of a channel block on the surface of the substrate, a reagent having the monomer A flows through or is placed in all or some of the channel(s). The channels provide fluid contact to the first selected regions, thereby binding the monomer A on the substrate directly or indirectly (via a spacer) in the first selected regions.

Thereafter, a monomer B is coupled to second selected regions, some of which may be included among the first selected regions. The second selected regions will be in fluid contact with a second flow channel(s) through translation, rotation, or

replacement of the channel block on the surface of the substrate; through opening or closing a selected valve; or through deposition of a layer of chemical or photoresist. If necessary, a step is performed for activating at least the second regions. Thereafter, the monomer B is flowed through or placed in the second flow channel(s), binding monomer
5 B at the second selected locations. In this particular example, the resulting sequences bound to the substrate at this stage of processing will be, for example, A, B, and AB. The process is repeated to form a vast array of sequences of desired length at known locations on the substrate.

After the substrate is activated, monomer A can be flowed through some
10 of the channels, monomer B can be flowed through other channels, a monomer C can be flowed through still other channels, etc. In this manner, many or all of the reaction regions are reacted with a monomer before the channel block must be moved or the substrate must be washed and/or reactivated. By making use of many or all of the available reaction regions simultaneously, the number of washing and activation steps can
15 be minimized.

One of skill in the art will recognize that there are alternative methods of forming channels or otherwise protecting a portion of the surface of the substrate. For example, according to some embodiments, a protective coating such as a hydrophilic or hydrophobic coating (depending upon the nature of the solvent) is utilized over portions
20 of the substrate to be protected, sometimes in combination with materials that facilitate wetting by the reactant solution in other regions. In this manner, the flowing solutions are further prevented from passing outside of their designated flow paths.

The "spotting" methods of preparing nucleic acid libraries can be implemented in much the same manner as the flow channel methods. For example, a
25 monomer A can be delivered to and coupled with a first group of reaction regions which have been appropriately activated. Thereafter, a monomer B can be delivered to and reacted with a second group of activated reaction regions. Unlike the flow channel embodiments described above, reactants are delivered by directly depositing (rather than flowing) relatively small quantities of them in selected regions. In some steps, of course,
30 the entire substrate surface can be sprayed or otherwise coated with a solution. In preferred embodiments, a dispenser moves from region to region, depositing only as much monomer as necessary at each stop. Typical dispensers include a micropipette to deliver the monomer solution to the substrate and a robotic system to control the position of the micropipette with respect to the substrate, or an ink-jet printer. In other

embodiments, the dispenser includes a series of tubes, a manifold, an array of pipettes, or the like so that various reagents can be delivered to the reaction regions simultaneously. Still other spotting methods are described in PCT/US99/00730.

5 *Pin-Based Methods*

Another method which is useful for the preparation of nucleic acid arrays and libraries involves "pin based synthesis." This method is described in detail in U.S. Pat. No. 5,288,514. The method utilizes a substrate having a plurality of pins or other extensions. The pins are each inserted simultaneously into individual reagent containers
10 in a tray. In a common embodiment, an array of 96 pins/containers is utilized.

Each tray is filled with a particular reagent for coupling in a particular chemical reaction on an individual pin. Accordingly, the trays will often contain different reagents. Since the chemistry disclosed herein has been established such that a relatively similar set of reaction conditions may be utilized to perform each of the reactions, it
15 becomes possible to conduct multiple chemical coupling steps simultaneously. In the first step of the process the invention provides for the use of substrate(s) on which the chemical coupling steps are conducted. The substrate is optionally provided with a spacer having active sites. In the particular case of nucleic acids, for example, the spacer may be selected from a wide variety of molecules which can be used in organic environments
20 associated with synthesis as well as aqueous environments associated with binding studies. Examples of suitable spacers are polyethyleneglycols, dicarboxylic acids, polyamines and alkylenes, substituted with, for example, methoxy and ethoxy groups. Additionally, the spacers will have an active site on the distal end. The active sites are optionally protected initially by protecting groups. Among a wide variety of protecting
25 groups which are useful are FMOC, BOC, t-butyl esters, t-butyl ethers, and the like. Various exemplary protecting groups are described in, for example, Atherton *et al.*, SOLID PHASE PEPTIDE SYNTHESIS, IRL Press (1989). In some embodiments, the spacer may provide for a cleavable function by way of, for example, exposure to acid or base.

30 *Solid supports*

Solid supports used in the present invention include any of a variety of fixed organizational support matrices. In some embodiments, the support is substantially planar. In some embodiments, the support may be physically separated into regions, for example, with trenches, grooves, wells and the like. Examples of supports include slides,

beads and solid chips. Additionally, the solid supports may be, for example, biological, nonbiological, organic, inorganic, or a combination thereof, and may be in forms including particles, strands, gels, sheets, tubing, spheres, containers, capillaries, pads, slices, films, plates, and slides depending upon the intended use.

5 Supports having a surface to which arrays of nucleic acids are attached are also referred to herein as "biological chips". The support is preferably, silica or glass, and can have the thickness of a microscope slide or glass cover slip. Supports that are transparent to light are useful when the assay involves optical detection, as described, e.g., in U.S. Patent No. 5,545,531. Other useful supports include Langmuir Blodgett film,
10 germanium, (poly)tetrafluorethylene, polystyrene, (poly)vinylidenedifluoride, polycarbonate, gallium arsenide, gallium phosphide, silicon oxide, silicon nitride, and combinations thereof. In one embodiment, the support is a flat glass or single crystal silica surface with relief features less than about 10 Angstroms.

The surfaces on the solid supports will usually, but not always, be
15 composed of the same material as the substrate. Thus, the surface may comprise any number of materials, including polymers, plastics, resins, polysaccharides, silica or silica based materials, carbon, metals, inorganic glasses, membranes, or any of the above-listed substrate materials. Preferably, the surface will contain reactive groups, such as carboxyl, amino, and hydroxyl. In one embodiment, the surface is optically transparent and will
20 have surface Si-OH functionalities such as are found on silica surfaces. In other embodiments, the surface will be coated with functionalized silicon compounds (see, for example, U.S. Patent No. 5,919,523).

Surface Density

25 The nucleic acid arrays described herein can have any number of nucleic acid sequences selected for different applications. Typically, there may be, for example, about 100 or more, or in some embodiments, more than 10^5 or 10^8 . In one embodiment, the surface comprises at least 100 probe nucleic acids each preferably having a different sequence, each probe contained in an area of less than about 0.1 cm^2 , or, for example,
30 between about $1 / \text{mm}^2$ and $10,000 / \text{mm}^2$, and each probe nucleic acid having a defined sequence and location on the surface. In one embodiment, at least 1,000 different nucleic acids are provided on the surface, wherein each nucleic acid is contained within an area less than about 10^{-3} cm^2 , as described, for example, in U.S. Patent No. 5,510,270.

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Arrays of nucleic acids for use in gene expression monitoring are described in PCT WO 97/10365, the disclosure of which is incorporated herein. In one embodiment, arrays of nucleic acid probes are immobilized on a surface, wherein the array comprises more than 100 different nucleic acids and wherein each different nucleic acid is localized in a predetermined area of the surface, and the density of the different nucleic acids is greater than about 60 different nucleic acids per 1 cm².

Arrays of nucleic acids immobilized on a surface which may be used also are described in detail in U.S. Patent No. 5,744,305, the disclosure of which is incorporated herein. As disclosed therein, on a substrate, nucleic acids with different sequences are immobilized each in a known area on a surface. For example, 10, 50, 60, 100, 10³, 10⁴, 10⁵, 10⁶, 10⁷, or 10⁸ different monomer sequences may be provided on the substrate. The nucleic acids of a particular sequence are provided within a known region of a substrate, having a surface area, for example, of about 1 cm² to 10⁻¹⁰ cm². In some embodiments, the regions have areas of less than about 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹, or 10⁻¹⁰ cm². For example, in one embodiment, there is provided a planar, non-porous support having at least a first surface, and a plurality of different nucleic acids attached to the first surface at a density exceeding about 400 different nucleic acids/cm², wherein each of the different nucleic acids is attached to the surface of the solid support in a different known region, has a different determinable sequence, and is, for example, at least 4 nucleotides in length. The nucleic acids may be, for example, about 4 to 20 nucleotides in length. The number of different nucleic acids may be, for example, 1000 or more. In the embodiment where polynucleotides of a known chemical sequence are synthesized at known locations on a substrate, and binding of a complementary nucleotide is detected, and wherein a fluorescent label is detected, detection may be implemented by directing light to relatively small and precisely known locations on the substrate. For example, the substrate is placed in a microscope detection apparatus for identification of locations where binding takes place. The microscope detection apparatus includes a monochromatic or polychromatic light source for directing light at the substrate, means for detecting fluoresced light from the substrate, and means for determining a location of the fluoresced light. The means for detecting light fluoresced on the substrate may in some embodiments include a photon counter. The means for determining a location of the fluoresced light may include an x/y translation table for the substrate. Translation of the substrate and data collection are recorded and managed by

an appropriately programmed digital computer, as described in U.S. Patent No. 5,510,270.

Applications Using Nucleic Acid Arrays

5 The methods and compositions described herein may be used in a range of applications including biomedical and genetic research as well as clinical diagnostics. Arrays of polymers such as nucleic acids may be screened for specific binding to a target, such as a complementary nucleotide, for example, in screening studies for determination of binding affinity and in diagnostic assays. In one embodiment, sequencing of
10 polynucleotides can be conducted, as disclosed in U.S. Patent No. 5,547,839. The nucleic acid arrays may be used in many other applications including detection of genetic diseases such as cystic fibrosis, diabetes, and acquired diseases such as cancer, as disclosed in U.S. Patent Application Ser. No. 08/143,312. Genetic mutations may be detected by sequencing by hybridization. In one embodiment, genetic markers may be
15 sequenced and mapped using Type-II restriction endonucleases as disclosed in U.S. Patent No. 5,710,000.

Other applications include chip based genotyping, species identification and phenotypic characterization, as described in U.S. Patent Application Serial No. 08/797,812, filed February 7, 1997, and U.S. Application Serial No. 08/629,031, filed
20 April 8, 1996. Still other applications are described in U.S. Patent No. 5,800,992.

Gene expression may be monitored by hybridization of large numbers of mRNAs in parallel using high density arrays of nucleic acids in cells, such as in microorganisms such as yeast, as described in Lockhart *et al.*, *Nature Biotechnology*,
25 **14**:1675-1680 (1996). Bacterial transcript imaging by hybridization of total RNA to nucleic acid arrays may be conducted as described in Saizieu *et al.*, *Nature Biotechnology*, **16**:45-48 (1998). Accessing genetic information using high density DNA arrays is further described in Chee, *Science* 274:610-614 (1996).

Still other methods for screening target molecules for specific binding to arrays of polymers, such as nucleic acids, immobilized on a solid substrate, are disclosed,
30 for example, in U.S. Patent No. 5,510,270. The fabrication of arrays of polymers, such as nucleic acids, on a solid substrate, and methods of use of the arrays in different assays, are also described in: U.S. Patent Nos. 5,677,195, 5,624,711, 5,599,695, 5,445,934, 5,451,683, 5,424,186, 5,412,087, 5,405,783, 5,384,261, 5,252,743 and 5,143,854; PCT WO 92/10092; and U.S. Application No. 08/388,321, filed February 14, 1995.

Devices for concurrently processing multiple biological chip assays are useful for each of the applications described above (see, for example, U.S. Patent No. 5,545,531). Methods and systems for detecting a labeled marker on a sample on a solid support, wherein the labeled material emits radiation at a wavelength that is different from the excitation wavelength, which radiation is collected by collection optics and imaged onto a detector which generates an image of the sample, are disclosed in U.S. Patent No. 5,578,832. These methods permit a highly sensitive and resolved image to be obtained at high speed. Methods and apparatus for detection of fluorescently labeled materials are further described in U.S. Patent Nos. 5,631,734 and 5,324,633.

Preferred Embodiments

In view of the technologies provided above, the present invention provides in one preferred embodiment, a method of preparing a nucleic acid array on a support, wherein each nucleic acid occupies a separate known region of the support and the nucleic acids are synthesized using the steps:

- (a) activating a region of the support;
- (b) attaching a nucleotide to a first region, the nucleotide having a masked reactive site linked to a protecting group;
- (c) repeating steps (a) and (b) on other regions of the support whereby each of the other regions has bound thereto another nucleotide comprising a masked reactive site link to a protecting group, wherein the another nucleotide may be the same or different from that used in step (b);
- (d) removing the protecting group from one of the nucleotides bound to one of the regions of the support to provide a region bearing a nucleotide having an unmasked reactive site;
- (e) binding an additional nucleotide to the nucleotide with an unmasked reactive site;
- (f) repeating steps (d) and (e) on regions of the support until a desired plurality of nucleic acids is synthesized, each nucleic acid occupying separate known regions of the support;

wherein the surface of the substrate is maintained in a position which is vertical or within about 30 degrees of vertical during the attaching and binding steps, and

wherein the substrate is rotated around an axis perpendicular to the surface by an amount of from about 20 degrees to about 180 degrees, the rotating being done prior to, coincident with or subsequent to at least one of the attaching or binding steps.

Preferably, the "activating" of step (a) is carried out using a channel block or photolithography technique, more preferably a photolithography technique. The "attaching" of step (b) is typically carried out using chemical means to provide a covalent bond between the nucleotide and a surface functional group present in the first region. In some embodiments, the surface functional group will be a group present on a nucleotide or nucleic acid that is already attached to the solid support. For example, nucleic acid arrays can be prepared using a solid support having a surface coated with poly-A nucleic acids to provide suitable spacing between the surface of the support and the nucleic acids that will be used in subsequent hybridization assays. Accordingly, the "attaching" can be, for example, by formation of a covalent bond between surface Si-OH groups and a group present on the first nucleotide of a nascent nucleic acid chain, or by formation of a covalent bond between groups present in a support-bound nucleic acid and a group present on the first nucleotide of a nascent nucleic acid. Typically, the groups present on nucleic acids which are used in covalent bond formation are the 3' - or 5-hydroxyl groups in the sugar portion of the molecule, or phosphate groups attached thereto.

The nucleotides used in this and other aspects of the present invention will typically be the naturally-occurring nucleotides, derived from, for example, adenosine, guanosine, uridine, cytidine and thymidine. In certain embodiments, however, nucleotide analogs or derivatives will be used (e.g., those nucleosides or nucleotides having protecting groups on either the base portion or sugar portion of the molecule, or having attached or incorporated labels, or isosteric replacements which result in monomers that behave in either a synthetic or physiological environment in a manner similar to the parent monomer). The nucleotides will typically have a protecting group which is linked to, and masks, a reactive group on the nucleotide. A variety of protecting groups are useful in the invention and can be selected depending on the synthesis techniques employed. For example, channel block methods can use acid- or base-cleavable protecting groups to mask a hydroxyl group in a nucleotide. After the nucleotide is attached to the support or growing nucleic acid, the protecting group can be removed by flowing an acid or base solution through an appropriate channel on the support.

Similarly, photolithography techniques can use photoremovable protecting groups. Some classes of photoremovable protecting groups include 6-nitroveratryl (NV),

6-nitropiperonyl (NP), methyl-6-nitroveratryl (MeNV), methyl-6-nitropiperonyl (MeNP), and 1-pyrenylmethyl (PyR), which are used for protecting the carboxyl terminus of an amino acid or the hydroxyl group of a nucleotide, for example. 6-nitroveratryloxycarbonyl (NVOC), 6-nitropiperonyloxycarbonyl (NPOC), methyl-6-nitroveratryloxycarbonyl (MeNVOC), methyl-6-nitropiperonyloxycarbonyl (MeNPOC), 1-pyrenylmethyloxycarbonyl (PyROC), which are used to protect the amino terminus of an amino acid are also preferred. Clearly, many photosensitive protecting groups are suitable for use in the present invention (see, U.S. Patent No. 5,489,678 and PCT WO 94/10128).

Additional photoremovable protecting groups such as 5'-O-pyrenylmethyloxy carbonyl (PYMOC) and methyl-6-nitropiperonyloxycarbonyl (MeNPOC) have been described in the copending U.S. Patent Application Ser. No. 08/630,148, filed April 10, 1996, the contents of which are hereby incorporated by reference.

In addition to the above-described protecting groups, the present invention employs protecting groups, such as the 5'-X-2'-deoxythymidine 2-cyanoethyl 3'-N,N-diisopropylphosphoramidites in various solvents. In these protecting groups, X may represent the following photolabile groups: ((α -methyl-2-nitropiperonyl)-oxy)carbonyl (MeNPOC), ((Phenacyl)-oxy)carbonyl (PAOC), O-(9-phenylxanthen-9-yl) (PIXYL), and ((2-methylene-9,10-anthraquinone)-oxy)carbonyl (MAQOC).

Various methods for generating protected monomers have been described by the U.S. Patent No. 5,744,305, which is incorporated by reference. Detailed methods for using photoremovable protecting groups are described in the U.S. Patent No. 5,424,186, which is also hereby incorporated by reference.

The removal rate of the protecting groups depends on the wavelength and intensity of the incident radiation, as well as the physical and chemical properties of the protecting group itself. Preferred protecting groups are removed at a faster rate and with a lower intensity of radiation. For example, at a given set of conditions, MeNVOC and MeNPOC are photolytically removed faster than their unsubstituted parent compounds, NVOC and NPOC, respectively.

In addition to the above-described references, photocleavable protecting groups and methods of using such photocleavable protecting groups for polymer synthesis have been described in the copending applications 08/630,148 (filed April 10, 1996) and 08/812,005 (filed March 5, 1997) which are incorporated by reference herein.

Step (c) provides that steps (a) and (b) can be repeated to attach nucleotides to other regions of the solid support.

One of skill in the art will appreciate that steps (a) and (b) can be repeated a number of times to produce a solid support having a layer of attached nucleotides.

5 Preferably, each attached nucleotide is in a known position.

In subsequent steps (d), (e) and (f), the protecting group is removed from one of the nucleotides to reveal a reactive site on the nucleotide. Thereafter, an additional nucleotide (optionally having a masked reactive site attached to a protecting group) is attached to the support-bound nucleotide. As above, these steps can be repeated to
10 selectively attach or bind an additional nucleotide to any of the support-bound nucleotides. Still further, the steps of deprotecting and attaching an additional nucleotide can be carried out on the newly added nucleotides to continue the synthesis of the nascent nucleic acid.

As noted above, the above steps are preferably carried out in combination
15 with substrate rotation. In one group of embodiments, a wafer (or solid support) is divided into 49 synthesis zones (chips) and placed in a substantially horizontal position in an irradiation chamber. Portions of the chips are illuminated through a photolithography mask to activate known regions of the chips. The wafer is then transferred to a synthesis apparatus wherein the wafer is held in a substantially vertical position and a flowcell
20 having certain reagents is attached to the wafer. For wafers that are square, the position is shown in Figure 1 (having a vertex pointing down). After the wafer is contacted with the desired reagents and attaching or binding has taken place, excess reagents can be removed by washing and the wafer can be transferred to the irradiation (or activation) chamber. Again, known regions of the wafer are illuminated to activate those regions. The wafer is
25 again transferred to the synthesis apparatus and rotated prior to contacting the wafer with the desired reagents. The process can be continued until an array having a desired number of attached oligonucleotides has been prepared.

In a further preferred embodiment, the preparing comprises:

a) removing a photoremovable protecting group from at least a first area
30 of a surface of a substrate, the substrate comprising immobilized nucleotides on the surface, and the nucleotides capped with a photoremovable protective group, without removing a photoremovable protecting group from at least a second area of the surface;

b) simultaneously contacting the first area and the second area of the surface with a first nucleotide to couple the first nucleotide to the immobilized

nucleotides in the first area, and not in the second area, the first nucleotide capped with a photoremovable protective group;

c) removing a photoremovable protecting group from at least a part of the first area of the surface and at least a part of the second area;

5 d) simultaneously contacting the first area and the second area of the surface with a second nucleotide to couple the second nucleotide to the immobilized nucleotides in at least a part of the first area and at least a part of the second area;

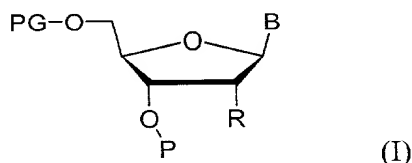
e) performing additional removing and nucleotide contacting and coupling steps so that a matrix array of at least 100 nucleic acids having different sequences is
10 formed on the support;

wherein the surface of said substrate is maintained in a position which is vertical or within about 30 degrees of vertical, and

wherein the substrate is rotated around an axis perpendicular to said surface by an amount of from about 20 degrees to about 180 degrees, said rotating being
15 done prior to, coincident with or subsequent to at least one of said attaching or binding steps.

In this embodiment of the invention, the steps of removing photoremovable protecting groups, coupling nucleotides to specific areas, removing protecting groups from the coupled nucleotides, and coupling additional nucleotides can
20 all be carried out as described in, for example, U.S. Patent No. 5,510,270, with the added feature that the substrate is rotated during the course of synthesis.

In still further preferred embodiments, the nucleoside phosphoramidite monomers used in the methods described above have the formula:



25 wherein B represents adenine, guanine, thymine, cytosine, uracil or analogs thereof; R is hydrogen, hydroxy, protected hydroxy, halogen or alkoxy; P is a phosphoramidite group; and PG is a photoremovable protected group.

In the group of emodiments using monomers of formula (I), B is preferably adenine, guanine, thymine, cytosine or uracil. More preferably, B is adenine,
30 guanine, thymine, or cytosine, and R is hydrogen. Still more preferably, the array prepared using the monomers above comprises at least 10 different nucleic acids, more

preferably at least 100 different nucleic acids, still more preferably at least 1000 different nucleic acids. Most preferably, the array comprises at least 10,000 to 100,000 or more different nucleic acids. Additionally, each different nucleic acid is in a region having an area of less than about 1 cm², more preferably less than about 1 mm².

5 In still other preferred embodiments, B is adenine, guanine, thymine, or cytosine; R is hydrogen.

In further preferred embodiments, B is adenine, guanine, thymine, or cytosine; R is hydrogen; and PG is MeNPOC.

10 In still further preferred embodiments, B is adenine, guanine, thymine, or cytosine; R is hydrogen; PG is MeNPOC, and P is -P(OCH₂CH₂CN)N(iPr)₂.

One of skill in the art will appreciate that the present invention can be readily modified to use protected nucleoside phosphoramidite monomers wherein the protecting group on the 5' hydroxy is acid or base removable. Such modifications will
15 render the invention applicable to other synthesis methodologies such as flow channel and spotting methods described in more detail above. Regardless of the array synthesis methods, substrate rotation provides an enhancement in both quantity and performance of the resulting chips or arrays.

20 EXAMPLES

Example 1

This example illustrates the improvement in oligonucleotide array
25 construction and performance that can be achieved using the methods described herein.

Test vehicles, checkerboard of the "213" and "Block 3" sequences were used to perform preliminary experiments. For each of the Checkerboard and Control Block 3 arrays, the oligonucleotides were synthesized in a 23-step process to form arrays of 20-mers. Fluorescein stain, 0.5 hour at 35o C hybridization assay was used to indicate
30 the variation between arrays synthesized. The results were then confirmed by synthesizing Gene expression arrays (e.g., Mu11K), a 74-step process. The standard QC assay, Phycoerythrin/streptavidan (SAPE) stain, 18 hours at 45 o C hybridization assay, was used to evaluate the arrays. The average difference of the "Block3" and "Bio C" were analyzed on the Gene Expression probe arrays.

In each step, a square wafer is placed in an illumination chamber and light is directed at the wafer through a mask to selectively deprotect and activate specific sites on the wafers. The wafers are then transferred to a synthesis chamber wherein the wafers are held in a vertical position with one corner of the square wafer pointing down. A flowcell is contacted with the wafer and reagents are directed onto the wafer from the bottom to the top. Prior to each step in the synthesis chamber, the wafers are rotated in multiples of 90° (e.g, 90°, 180°, 270°) so that one corner of the wafer is always pointed downward.

Table 1

Type of Array	Number of wafers	Coefficient of Variation (%)	Top to Bottom Ratio	Rotation type
Checkerboard 213 – control	4	17%, 21%, 19%, 20%	1.4, 1.5, 1.4, 1.4	
Checkerboard 213– rotated	2	6%, 6%	1.0, 1.0	90 degrees
Checkerboard Block 3 – control	1	8%	1.4	
Checkerboard Block 3 – rotated	1	2%	1.0	90 degrees
Murine gene expression – Block 3 Control	2	55%, 77%	2.9, 5.0	
Murine gene expression – Block 3 Rotated	1	19%	1.2	random 90 degrees
Murine gene expression – Bio C-Control	2	68%, 78%	3.7, 4.9	
Murine gene expression - Bio C – Rotated	1	35%	1.7	random 90 degrees

As can be seen from the table of data above, rotating the wafers during synthesis led to a significant reduction in intra wafer variability of from about 50% (68% CV versus 35% CV) to about 75% (8% CV versus 2% CV).

Figure 2 is a graph that illustrates the average difference in control versus rotated arrays. In this experiment, lot 95296 as a control exhibited Block 3 average

differences of about 50-150. The rotated lots, 95297 and 95299, exhibited average differences of about 45-85 and about 40-70, respectively, indicating the reduced variability for rotated lots.

Figure 3 provides an analysis of chips from different portions of each wafer. In this graph, b = bottom, c = center, t = top, l = left and r = right. As can be seen in Figure 3, the control wafer (296) exhibited markedly wider ranges of average difference, while the rotated wafers (297 and 299) provided more consistent results between the portions of the wafer.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.